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13. ABSTRACT (Maximum 200 Words)

The androgen-signaling pathway is important in the growth and progression of prostate cancer. The growthpromoting effects of androgen are mediated mostly through the androgen receptor (AR). PI3K/Akt plays a critical role in prostate cancer cell growth and survival. It has been shown that the effect of PI3K/Akt in prostate cells is mediated through androgen signaling. The PI3K inhibitor, LY294002, and a tumor suppressor, PTEN, negatively regulate the PI3K/Akt pathway and repress AR activity. However, the molecular mechanisms whereby PI3K/Akt and PTEN regulate the androgen pathway are currently unclear. The proposed studies examine whether β–catenin is a major downstream effector of the PI3K/Akt and PTEN pathways in androgen-mediated prostate cell growth. Several sets of *in vivo* and *in vitro* experiments have been performed to further test our hypothesis during this funding year. Successful completion of the proposed studies should provide fresh insight into the novel link between the PI3K, Wnt, and androgen pathways, which may help us to identify new pathways that can be targeted for prostate cancer treatment.

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INTRODUCTION:

Prostate cancer is the most common malignancy in men and the second leading cause of cancer death in the United States (Landis et al., 1999). The androgen signaling pathway, which is mainly mediated through the androgen receptor (AR), is important for the normal and neoplastic development of prostate cells (Balk, 2002; Gelmann, 2002). Androgen ablation is an effective treatment for the majority of advanced prostate cancer patients (Kyprianou and Isaacs, 1988). The phosphatidylinositol 3-kinase (PI3K) consists of regulatory (p85) and catalytic (p110) subunits which participate in multiple cellular processes, including cell growth, transformation, differentiation, and survival (Carpenter and Cantley, 1996). PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene acts as an inhibitor of the PI3K to hydrolyze the lipid products of PI3K (Cantley and Neel, 1999). Loss of PTEN in prostate cancer cells results in constitutive activation of enzymes downstream of PI3K, including the Akt protein-Ser/Thr kinase (Li et al., 1997). PI3K/Akt have been shown to promote prostate cancer cell survival and growth via enhancing AR-mediated transcription (Li et al., 2001). Both PTEN and the PI3K inhibitor, LY294002, negatively regulate this process (Li et al., 2001; Wen et al., 2000). Although several potential mechanisms have been suggested for this crosstalk, the precise molecular basis by which PI3K/AKT and PTEN regulate AR-mediated transcription is currently unclear. In our previous studies, we have shown that the crosstalk between the androgen and PI3K/Akt pathways is mediated through modulation of the PI3K/Akt downstream effector, GSK3β (glycogen synthase kinase 3β). Its inactivation by phosphorylation results in increased nuclear levels of β-catenin, leads to the augmentation of AR activity. In the past year, we further investigated the regulation of β-catenin by PI3K/Akt in prostate cancer cells, and the role of Wnt/β-catenin in the tumorigenesis of prostate cancer.

BODY:

Previous studies have shown that PI3K/Akt and PTEN can modulate androgen-induced cell growth and AR-mediated transcription in prostate cancer cells (Li et al., 2001; Wen et al., 2000). The data suggested a potential link between the PI3K/Akt and androgen pathways. However, the precise molecular basis by which PI3K/AKT and PTEN regulate AR-mediated transcription remains unclear. It has been shown that the cytoplasmic β-catenins are tightly regulated by the destruction complex, which includes APC, GSK3β, and Axin. When these components are altered, by mutation, aberrant expression of the proteins, or inactivation by protein modulation, cellular levels of β-catenins are increased and the proteins can be translocated into the nuclei. In prostate cancer cells, the change may lead the enhancement of AR-mediated transcription and cell growth and survival. Four specific objectives were proposed in the original proposal to further characterize the role and the regulation of β-catenin in prostate cancer cells.

Objective 1. Determine the role of PI3K pathway in β-catenin mediated cell growth.

The major hypothesis tested in this aim is whether the augmentation of AR transcriptional activity by β–catenin is regulated by the PI3K inhibitor. We have used tetracycline-inducible LNCaP cell lines, which stably express wild type or mutated β–catenin genes. In the past years, we focused on generating these cell lines to test their inducibilities. Currently, we are performing both cell proliferation and colony formation assays. Cells infected with pRev-TRE-Flag-β-catenin/wt or pRev-TRE-Flag-β-catenin/mut were grown in medium with tetracycline-free FBS or regular FBS (tetracycline +) in the presence of DHT. After overnight incubation, cells were pulsed for 6 hours with 200 μl/well of 10 μCi/ml of ^{3}H-Thymidine at 37°C. Cells were harvested and measured for ^{3}H-thymidine incorporation. We evaluated the effect of β–catenin by comparing induced and un-induced cells after androgen treatment. In our pilot experiments, we observed decreased incorporation in the cells transfected with wild type β–catenin after the induction (Figure 1). Currently, we are repeating the above experiments and verifying the level of the β-catenin protein in the above cells.

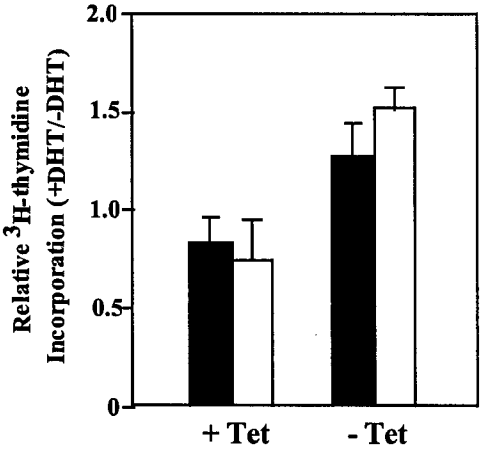

Figure 1. Tetracycline-inducible LNCaP cell lines stably express wild type or mutated β–catenin (S33F) genes were grown in medium with tetracycline-free FBS or regular FBS (tetracycline +) in the presence of DHT (1nM). After overnight incubation, cells were pulsed for 6 hours with 200 μl/well of 10 μCi/ml of ^{3}H-Thymidine at 37°C. Cells were harvested and measured for ^{3}H-thymidine incorporation. The relative incorporation rates represent the mean ±S.D. of three independent samples.

■ β–cat Wt □ β–cat Mut (S33F)

Objective 2: To determine whether LY294002 and PTEN regulate Tcf/LEF activity.

In the canonical pathway, the signaling pathway of Wnt is mediated through β–catenin binding to Tcf/LEF family members to activate the transcription of the downstream targets. The results from our preliminary studies showed that LY294002 and PTEN regulate the enhancement of β-catenin on the AR regulated promoter, PSA. However, it still remains unclear whether Tcf/LEF transcription factors are active in prostate cancer cells. For this reason, luciferase reporter containing an optimal LEF-binding site (TOPFlash) or mutated LEF-binding sites (FOPFlash) (Morin et al., 1997) were transfected alone or with a β-catenin expression vector into either AR-positive (LNCaP, LAPC4) or AR-negative (PC3, DU145) prostate cancer cell lines. As shown in Figure 2, no significant induction of TOPFlash activity was observed in all of cell lines either in presence or absence of exogenous β-catenin. Our data are consistent with the previous reports and suggests that there is no endogenous TCF/LEF activity in those prostate cancer cell lines (Truica et al., 2001). Currently, we are examining more prostate cancer cell lines. In addition, we will use several human tumor cell lines, which were shown endogenous Tcf/LEF activities, in our future transfection experiments.

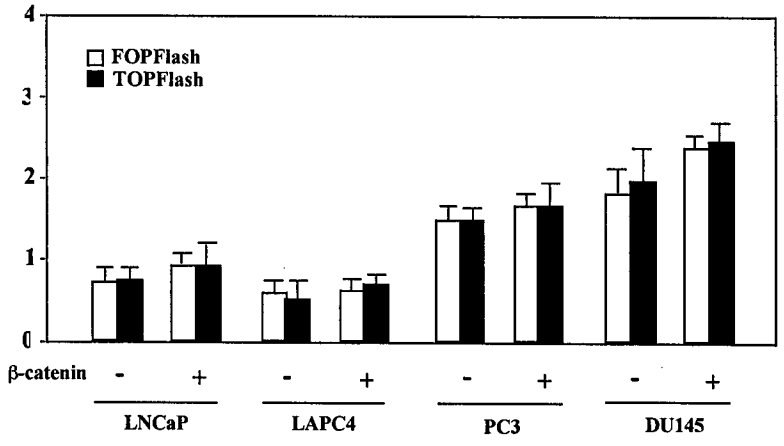

Figure 2. TCF/LEF is inactive in prostate cancer cell lines. Transient transfections were performed in LNCaP, LAPC4, PC3, and DU145 cells with 100 ng of TOPFlash or FOPFlash reporter and 10 ng of β-catenin and 25 ng of pcDNA3-β-gal. The cells were incubated for 36-42 hours after transfections. Cell lysates were measured for luciferase and β-gal activities. The data represent the mean ± S.D. of three independent samples.

Objective 3: Determine whether IGF is involved in β-catenin mediated enhancement of AR activity. We continue to test whether IGF-1 (insulin-like growth factor-1) induced AR activation is mediated through the

β-catenin. Previously, we have shown that IGF-1 enhances AR-mediated transcriptional activity. Here, we examined the ability of either the wild-type IGF-1R or a constitutively activated receptor, IGF-1R-NM1, to enhance AR-mediated transcription. In cells cultured with 5% fetal calf serum, both the wild type IGF-1 and IGF-1R-NM1 augmented AR-mediated transcription from the PSA promoter in a dose-dependent manner ($p<0.05$) (Fig. 3A). The IGF-1R-NM1 receptor showed a stronger augmentation of AR-mediated transcription than the wild-type receptor both in the absence and presence of androgens ($p<0.05$). These results provide an additional line of evidence that IGF-1 signaling plays a role in regulating AR-mediated transcription.

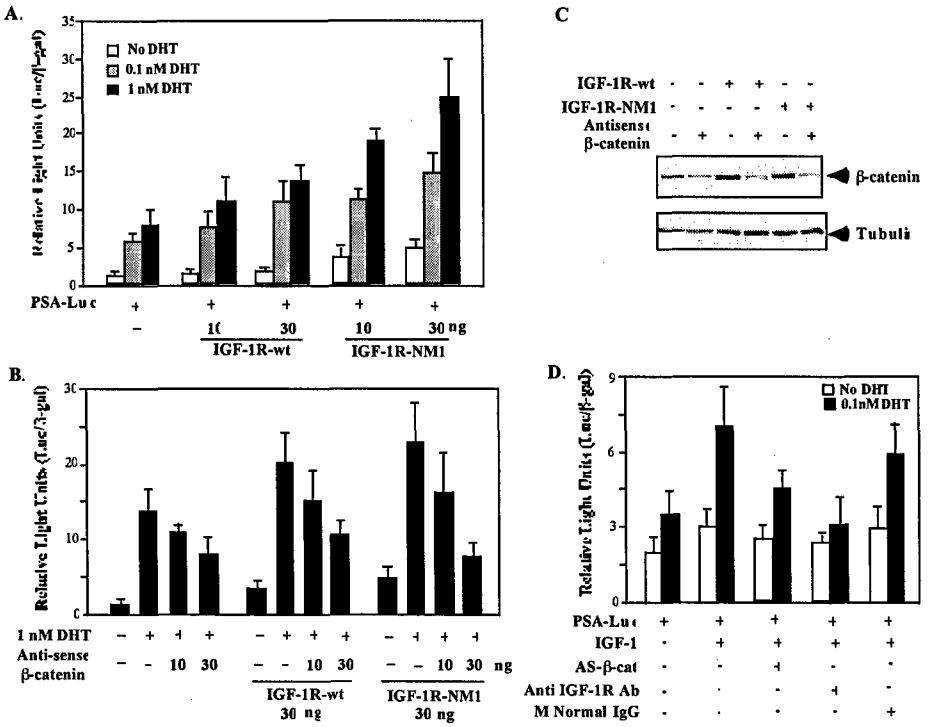

Figure 3. Overexpression of IGF-1 receptors enhances AR-mediated transcription. (A) Transient transfections were performed in LNCaP cells with 100 ng of PSA7kb-luc reporter and 10 or 30 ng of wild type (wt) or a constitutively active mutant (NM1) of IGF-1 receptor and 25 ng of pcDNA3-β-gal. The cells were incubated in T-medium with 5% charcoal-stripped FBS for 12 hours, and then were treated with different concentrations of DHT for 18 hours. Cell lysates were measured for luciferase and β-gal activities. The data represent the mean ± S.D. of three independent samples. (B) The transfection experiments were repeated in LNCaP cells under the same conditions as described above. Different amounts of the antisense β-catenin plasmid were co-transfected into the cells. The relative light units (RLU) from individual transfections were normalized by measurement of β-gal activity expressed from a co-transfected plasmid in the same samples. (C) The levels of cytosolic β-catenin were measured by Western blotting from LNCaP transfected with 30 ng of the IGF-1 receptors and 30 ng of antisense β-catenin plasmids (AS-β-cat). (D) One hundred ng of PSA7kb-luc reporter with or without 30 ng of the β-catenin antisense plasmids was transfected into LNCaP cells. The cells were cultured in T-medium with or without 100 ng/ml of IGF-1 in the presence or absence of DHT for 28-32 hr. One μg per ml of the IGF-1R antibody (Cat#: GR11, CalBiochem, San Diego, CA) or mouse normal IgG was added into cells 6 hr after transfection. Luciferase and β-gal) activities were measured as described above.

To further demonstrate that the enhancement of AR activity by IGF-1 receptors was mediated by β–catenin, we repeated the above experiments with an antisense construct of β–catenin (Yang et al., 2002). As shown in Figure 3B, co-transfection of the antisense β-catenin plasmid represses the enhancement of AR

by both the wild type and the mutant IGF-1R. With 30 ng of the antisense construct, AR activity was reduced 45% and 65% in cells transfected with the wild type and mutant IGF-1R ($p < 0.05$), respectively. To evaluate the effectiveness of β-catenin antisense constructs, we also measured the level of the cytosolic β-catenin protein in the above samples. As expected, the antisense β-catenin constructs reduced the levels of cytosolic β-catenin (Fig. 3C), which correlated with the reduction in AR transcriptional activity in the cells. To further demonstrate the role of β-catenin in IGF-1 induced AR activity, we examined whether the inhibition of β-catenin expression can affect IGF-1 induced AR activity by using the β-catenin antisense constructs. As shown in Figure 3D, the IGF-1 induced AR transactivation of the PSA promoter is abrogated by cotransfection with the β-catenin antisense constructs. In addition, we also demonstrated that the specific IGF-1R antibody, alpha IR3, effectively blocks the IGF-1 mediated AR activity. Taken together, the above results demonstrate a direct involvement of β-catenin in the IGF-1 signaling modulated, AR-mediated transcription.

It has been shown that IGF-1 enhances the stability of the β-catenin protein in human colorectal cancer cells (Playford et al., 2000). IGF-1 inhibits GSK3β by stimulating the phosphorylation of GSK3β (Stambolic and Woodgett, 1994; Sutherland et al., 1993). We therefore examined whether the IGF-1 enhancement of the stability of β-catenin in prostate cancer cells is mediated through GSK3β. Since LiCl has been shown to repress GSK3β (Stambolic et al., 1996), thereby stabilizing cellular β-catenin, we measured the cytosolic level of β-catenin in LNCaP cells treated with or without IGF-1. As observed previously, we saw an increase in β-catenin in cells treated with IGF-1 (Figure 4A). In the presence of 50 mM LiCl, the samples isolated from cells treated with 100 ng/ml of IGF-1 showed higher levels of β-catenin than ones not treated with LiCl. We conclude that inhibition of GSK3β can further enhance the levels of cellular β-catenin induced by IGF-1 in prostate cancer cells.

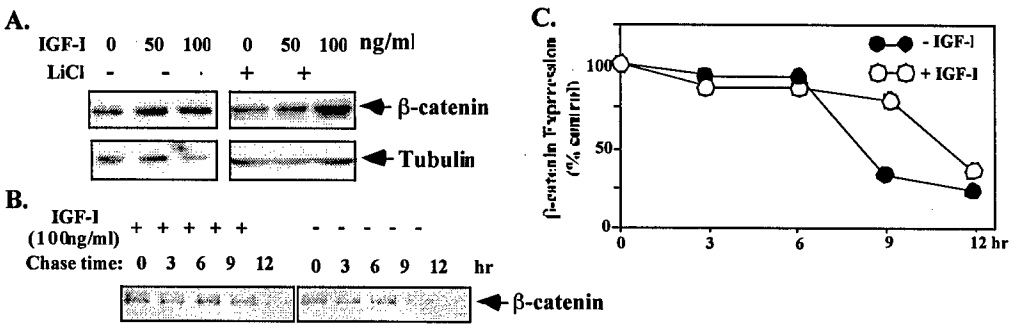

Figure 4. IGF-1 enhances the stability of β-catenin. (A) The cytosolic fraction was used to measure the free form of β-catenin in LNCaP cells treated with different concentration of IGF-1 in the presence or absence of LiCl. (B) LNCaP cells were pulsed with Tran^{35}S-label and chased with medium containing an excess of cold methionine/cysteine for the indicated times. The cytosolic fractions isolated from cells were immunoprecipitated for β-catenin. (C) The results were analyzed by densitometry and expressed graphically as a percentage of the value at 0 hour. The figure shows results of a single experiment, which was repeated once with similar results.

To demonstrate that the effect of IGF-1 was on the stability of β-catenin in prostate cancer cells, we performed pulse-chase experiments in LNCaP cells. Cells were pulsed with Tran^{35}S label and chased in the presence or absence of IGF-1 for 12 hours. ^{35}S-labeled β-catenin proteins were immunoprecipitated from the cytosolic fractions and analyzed by SDS-PAGE. As shown in Figure 4B, IGF-1 enhances the stability of β-

catenin, increasing its half-life from 9 to 12 hours. These results provide a direct line of evidence that IGF-1 indeed affects the stability of β-catenin in prostate cancer cells.

KEY RESEARCH ACCOMPLISHMENTS:

1) Generate and establish several LNCaP sublines stably transfected with the wild type and mutated β-catenin constructs.
2) Confirm TCF/LEF being transcriptionally inactive in four prostate cancer cell lines.
3) Further demonstrate that IGF-1 enhances the expression of PSA, a target gene of AR, in prostate cells by transient transfection assays.
4) Demonstrate that IGF-1 increases the level of cellular β-catenin in prostate cancer cells.

REPORTABLE OUTCOMES:

Publications:

Meletios Verras, Jeffrey Brown, Xiaomeng Li, Roel Nusse, Sun Z.J. (2004). Wnt3a Growth Factor Induces Androgen Receptor-mediated Transcription and Enhances Cell Growth in Human Prostate Cancer Cells. *Cancer Res.*, 64:8860-66.

Meletios Verras and Sun Z.J. (2005). Beta-catenin Is Involved in Insulin-Like Growth Factor 1-Mediated Transactivation of the Androgen Receptor. *Mol. Endo.*, 19:391-98.

Reagents:

LNCaP cell lines stably transfected with wild type or mutated β-catenin.

CONCLUSIONS:

Both Wnt/β-catenin and PI3/Akt pathways play a critical role in cell proliferation and survival. The major objective of this study is to determine the molecular mechanisms by which PI3K and PTEN regulate β-catenin in androgen signaling pathway in prostate cancer cells and the biological consequences of this regulation. In the past year, we performed several sets of experiments in order to achieve our goals. Particularly, we collected several solid lines of evidence showing that induction of AR-mediated transcription by IGF-1 is mediated through β-catenin. In addition, we also confirmed the observation by others that TCF/LEF family members are inactive in prostate cancer cells. Based on the observations, we started to investigate the non-canonical pathway in prostate cancer cells. We hope that through these efforts we can gain more information about the interaction between PI3K/Akt, IGF1, and androgen signaling pathways.

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Molecular Endocrinology 19(2):391–398

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β-Catenin Is Involved in Insulin-Like Growth Factor 1-Mediated Transactivation of the Androgen Receptor

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The androgen-signaling pathway is important for the growth and progression of prostate cancer cells. IGF-I and other polypeptide growth factors have been shown to be capable of induction of androgen receptor (AR) activation in the absence of, or at low levels of, ligand. It has been shown that IGF-I increases the cellular level of β-catenin, an AR coactivator. In this study, we performed several experiments to test whether β-catenin is involved in IGF-I-induced AR-mediated transcription. We demonstrate that IGF-I enhances the expression of endogenous *prostate-specific antigen,* an AR target gene, and elevates the level of cytoplasmic and nuclear β-catenin in prostate cancer cells. Transfection of either wild-type or a constitutively active mutant of the IGF-I receptor augments AR-mediated transcription. An antisense construct of β-catenin that decreases the cellular level of β-catenin can reduce IGF-1 receptor-mediated enhancement of AR activity. Moreover, using a pulse-chase experiment, we showed that IGF-I enhances the stability of β-catenin in prostate cancer cells. Our findings delineate a novel pathway for IGF-I in modulating androgen signaling through β-catenin. (*Molecular Endocrinology* 19: 391–398, 2005)

THE FACT THAT androgen ablation is an effective treatment for the majority of metastatic prostate cancer patients indicates that androgen plays an essential role in regulating the growth of prostate cancer cells (1). Unfortunately, most patients develop androgen-insensitive prostate cancer within 2 yr, for which there is currently no effective treatment. Failure of androgen ablation suggests that there are alterations in androgen signaling in the tumor cells. Multiple mechanisms by which prostate cancer cells progress to androgen-insensitive stages have been proposed (2).

Previous experiments have shown that androgen receptor (AR) can be activated in cells treated with polypeptide growth factors in the absence of, or at low levels of androgens (3). IGF-I is the most efficient growth factor capable of ligand-independent activation of AR. However, the mechanisms by which IGF-I or other growth factors regulates AR-mediated transcription in prostate cells remain unclear. Recently, Playford *et al.* (4) reported that IGF-I enhances tyrosine phosphorylation of β-catenin in human colorectal cancer cells, which results in dissociation of β-catenin from E-cadherin complexes at the cell membrane. Induction by IGF-I also increases the stability of the β-catenin protein (4). These results suggest a new link between β-catenin and IGF-I-mediated cell growth and transformation.

Recently, we and others (5–8) have demonstrated a specific protein-protein interaction between β-catenin and AR. Through the interaction, β-catenin augments AR-mediated transcription. β-Catenin plays a pivotal role in cadherin-based cell adhesion and in the Wnt signaling pathway [see the review by Polakis (9)]. Most β-catenin is located in the cell membrane where it is associated with the cytoplasmic region of E-cadherin, a transmembrane protein involved in homotypic cell-cell contacts (10). Smaller pools of β-catenin are located in both the nucleus and cytoplasm, where the protein mediates Wnt signaling. The wnt signal and E-cadherin can modulate the cellular level of β-catenin (6, 11). Accumulated β-catenin translocates into the nucleus and forms transcriptionally active complexes with Tcf/LEF (12) or the AR (5, 6). AR can modulate Tcf/LEF-mediated cellular effects by binding to limiting amounts of β-catenin, which may be critical during normal prostate development and tumor progression (13, 14).

Based on the facts that β-catenin is an AR coactivator and that IGF-I affects AR-mediated transcription, we investigated the molecular mechanism by which IGF-I enhances AR activity. Particularly, we addressed whether the activation of AR by IGF-I in prostate cells is modulated by the cellular level of β-catenin. In the human prostate cancer cell line, LNCaP, we show that

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Abbreviations: AR, Androgen receptor; ARE, androgen response element; DHT, dihydrotestosterone; FCS, fetal calf serum; β-gal, β-galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3β, glycogen synthase kinase-3β; H1, histone; IGF-1R, IGF-I receptor; PI3K, phosphoinositol 3-kinase; PSA, prostate-specific antigen.

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IGF-I enhances AR-mediated transcription at a low level of androgen, and that it increases the level of cellular β-catenin. Transfection of either wild-type or a constitutively active mutant of the IGF-I receptor (IGF-1R) augments AR-mediated transcription. Moreover, using an antisense construct of β-catenin, we further demonstrate that a decrease in the cellular level of β-catenin can reduce IGF-1R-mediated enhancement of AR activity. Furthermore, pulse-chase experiments demonstrate that IGF-I enhances the stability of the β-catenin protein in prostate cancer cells. These findings delineate a novel mechanism by which IGF-I modulates androgen signaling in prostate cells and provides fresh insight into the role of IGF-I in the development of androgen-insensitive prostate cancer.

RESULTS

IGF-I Enhances the Expression of Prostate-Specific Antigen (*PSA*), a Target Gene of AR, in Prostate Cells

Previous experiments showed that AR can be activated in cells treated with growth factors in the absence of, or at low levels of ligand (3). IGF-I is the most efficient growth factor capable of ligand-independent activation of AR. To evaluate the effect of IGF-I in a biologically relevant setting, we tested whether IGF-I regulates expression of the *PSA* gene, an endogenous AR target, in the AR-positive prostate cancer cell line, LNCaP. Using real-time PCR, we first measured transcripts of *PSA* in LNCaP cells treated with different amounts of IGF-I. In the presence of 0.1 nM dihydrotestosterone (DHT), *PSA* expression was increased approximately 20 or 35% in LNCaP cells treated with 30 or 100 ng/ml of IGF-I, respectively, over that found in cells not treated with IGF-I ($P < 0.05$) (Fig. 1A). However, in the absence of DHT, the expression of *PSA* was not significantly affected by IGF-I. To confirm this finding, we examined the expression of *PSA* by conventional Northern blotting. As observed in the real-time PCR assays, an increase in *PSA* transcripts was found in the cells treated with 30 or 100 ng/ml of IGF-I (Fig. 1B). Using β-actin as a reference gene, we showed an approximately 0.3- to 1-fold increase in *PSA* transcripts in the cells treated with 30 or 100 ng/ml of IGF-I, respectively (Fig. 1C). These results provide the first line of evidence that IGF-I is able to enhance endogenous AR-mediated transcription in prostate cancer cells in the presence of a low level of androgens.

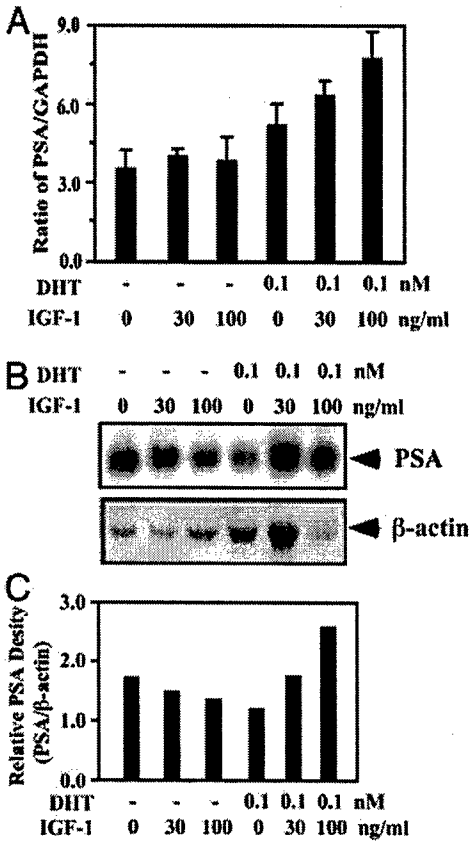

Fig. 1. IGF-I Enhances the Transcription of an Endogenous AR Target Gene, *PSA*

A, Expression levels of *PSA* mRNA were quantified using quantitative fluorescent real-time PCR. Total RNAs were isolated from LNCaP cells cultured in T-medium with or without 0.1 nM DHT, treated for 16 h with IGF-I or vehicle. RNA samples were first reverse-transcribed using random hexamers. Two specific primers selected from the regions around the translation initiation site or the stop codon of the *PSA* gene were used for amplification. PCR assays were performed with TaqMan PCR reagent Kits in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The levels of *PSA* mRNA were normalized by coamplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as described by the manufacturer (PE Applied Biosystems). B, Expression of the endogenous *PSA* transcripts was detected by a cDNA probe derived from the human *PSA* gene in LNCaP cells treated with IGF-I as described above. A β-actin probe was used to confirm equal RNA loading. C, Densitometry of the Northern blot was performed, and the relative numbers are reported as PSA/β-actin.

IGF-I Increases the Level of Cellular β-Catenin in Prostate Cancer Cells

It has been shown that IGF-I can elevate the cellular level of β-catenin in the human colon cancer cell line, C10 (4). β-Catenin has been demonstrated to be an AR coactivator (5, 6). To determine whether β-catenin is involved in IGF-I mediated AR transcription, we examined free cellular β-catenin in prostate cancer cells as described previously (4). As shown in Fig. 2A, there was no significant change in the amount of β-catenin in the cytoskeletal compartment (RIPA) of cells treated with IGF-I. However, there was a 2- to 4-fold increase in cytosolic β-catenin in cells treated with 50 or 100 ng of IGF-I (Digi), respectively. In contrast, the level of cytosolic tubulin, used as a

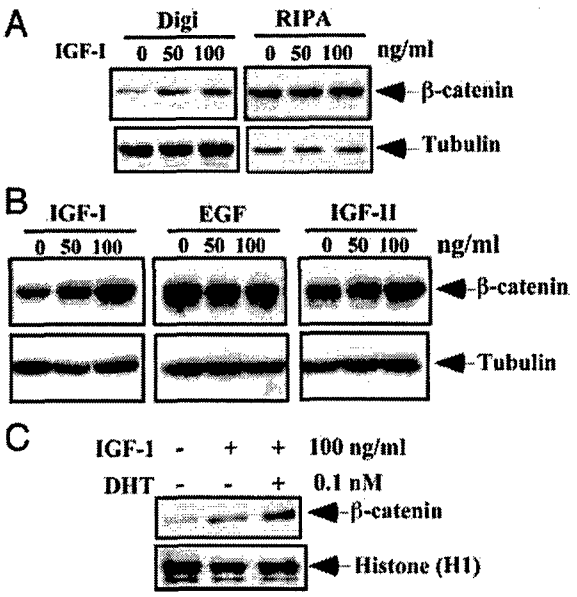

Fig. 2. IGF-I Signaling Enhances the Level of Free Cytosolic β-Catenin

A, Both cytosolic (Digi) and cytoskeletal fractions (RIPA) were prepared from LNCaP cells as described in *Materials and Methods*. Both β-catenin and tubulin proteins were analyzed by Western blotting with specific antibodies. B, Cytosolic fractions were isolated from LNCaP cells treated with different concentrations of growth factors. The levels of β-catenin were analyzed by Western blotting. Tubulin was used as a control for protein loading. C, The endogenous β-catenin proteins were analyzed in nuclear extracts isolated from LNCaP cells treated with or without IGF-I or DHT as labeled in the figure. The level of β-catenin was detected by Western blotting with a specific β-catenin antibody. H1, a nuclear protein, was used as a control for protein loading.

control, showed no significant difference in the treated and untreated cells.

To further test whether the effect of IGF-I on β-catenin is a specific event, we repeated the above experiments with LNCaP cells treated with different growth factors. As shown in the figure, a pronounced increase in cytosolic β-catenin was observed in cells treated with IGF-I but not with EGF (Fig. 2B). With 100 ng/ml of IGF-II, a slight change of β-catenin was also observed, suggesting a potential role of IGF-II in the regulation of cellular β-catenin (see *Discussion*). In addition, we also performed transient transfection experiments using the *PSA*-promoter/reporter in LNCaP cells in the presence of the above growth factors. We observed that IGF-I is one of the most efficient growth factors in modulating AR-mediated transcription (data not shown), which is consistent with the previous report by Culig *et al.* (15).

Next, we examined whether IGF-I directly affects the translocation of β-catenin to the nucleus. In the presence of 0.1 nM of DHT, IGF-I significantly increases the level of nuclear β-catenin in LNCaP cells, whereas it only affects β-catenin slightly in the absence of ligand (Fig. 2C). These data are consistent with our previous observation that IGF-I has a more pronounced effect in enhancing AR-mediated transcription in the presence of low level of androgens. The histone (H1) protein, used as a control, showed no change (Fig. 2C). Taken together, these results confirm the role of IGF-I in enhancing the translocation of β-catenin into the nucleus, which agree with the previous studies showing the similar effect of IGF-I in enhancing nuclear β-catenin in human colorectal cancer cells (4).

Overexpression of IGF-1R Enhances AR-Mediated Transcription

To confirm that IGF-I enhances AR activity, we tested the ability of either the wild-type IGF-1R or a constitutively activated receptor, IGF-1R-NM1, generated by deleting the entire extracellular domain of IGF-1R and fusing the remaining receptor (16), to enhance AR-mediated transcription. In cells cultured with 5% fetal calf serum, both the wild-type IGF-I and IGF-1R-NM1 augmented AR-mediated transcription from the *PSA* promoter in a dose-dependent manner ($P < 0.05$) (Fig. 3A). The IGF-1R-NM1 receptor showed a stronger augmentation of AR-mediated transcription than the wild-type receptor both in the absence and presence of androgens ($P < 0.05$). These results provide an additional line of evidence that IGF-I signaling plays a role in regulating AR-mediated transcription.

To further demonstrate that the enhancement of AR activity by IGF-I receptors was mediated by β-catenin, we repeated the above experiments with an antisense construct of β-catenin (6). As shown in Fig. 3B, cotransfection of the antisense β-catenin plasmid represses the enhancement of AR by both the wild-type and the mutant IGF-1R. With 30 ng of the antisense construct, AR activity was reduced 45% and 65% in cells transfected with the wild-type and mutant IGF-1R ($P < 0.05$), respectively. To evaluate the effectiveness of β-catenin antisense constructs, we also measured the level of the cytosolic β-catenin protein in the above samples. As expected, the antisense β-catenin constructs reduced the levels of cytosolic β-catenin (Fig. 3C), which correlated with the reduction in AR transcriptional activity in the cells. To further demonstrate the role of β-catenin in IGF-I-induced AR activity, we examined whether the inhibition of β-catenin expression can affect IGF-I-induced AR activity by using the β-catenin antisense constructs. As shown in Fig. 3D, the IGF-I-induced AR transactivation of the *PSA* promoter is abrogated by cotransfection with the β-catenin antisense constructs. In addition, we also demonstrate that the specific IGF-1R antibody, αIR3, effectively blocks the IGF-I mediated AR activity. Taken together, the above results demonstrate a direct involvement of β-catenin in the IGF-I signaling modulated, AR-mediated transcription.

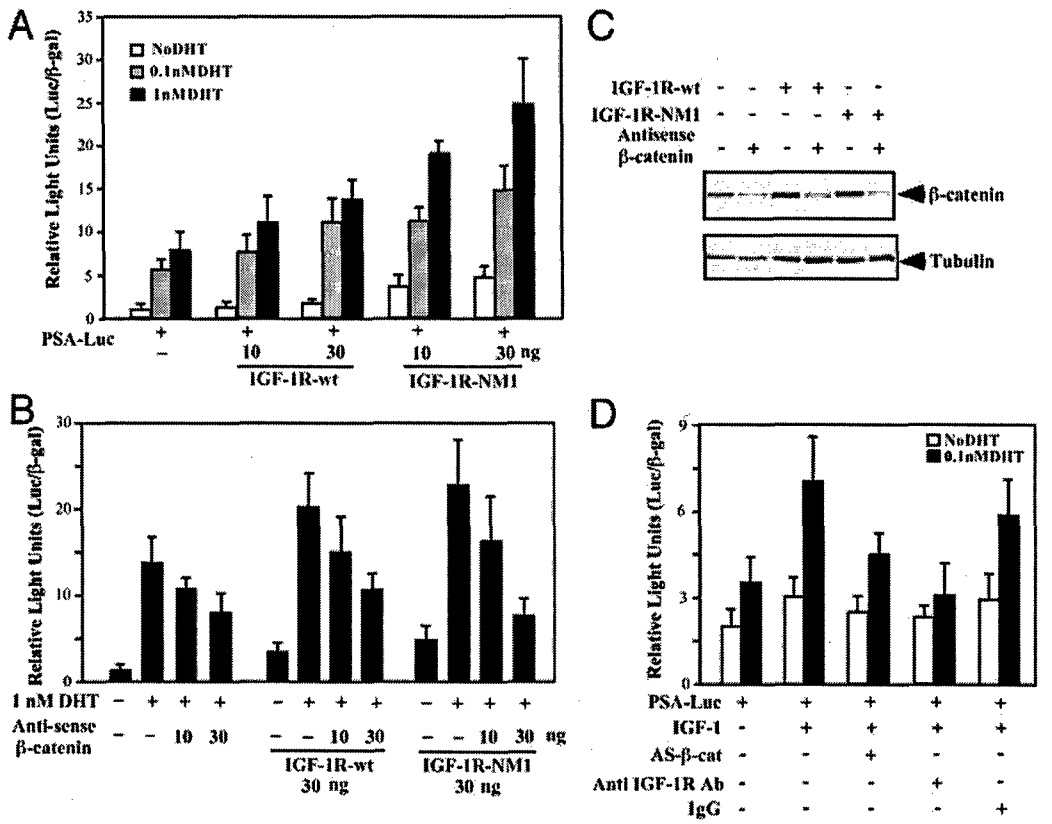

Fig. 3. Overexpression of IGF-I Receptors Enhances AR-Mediated Transcription

A, Transient transfections were performed in LNCaP cells with 100 ng of *PSA*7kb-luc reporter and 10 or 30 ng of wild type (wt) or a constitutively active mutant (NM1) of IGF-I receptor and 25 ng of pcDNA3-β-gal. The cells were incubated in T-medium with 5% charcoal-stripped FCS for 12 h, and then were treated with different concentrations of DHT for 18 h. Cell lysates were measured for luciferase and β-gal activities. The data represent the mean ± SD of three independent samples. B, The transfection experiments were repeated in LNCaP cells under the same conditions as described above. Different amounts of the antisense β-catenin plasmid were cotransfected into the cells. The relative light units (RLU) from individual transfections were normalized by measurement of β-gal activity expressed from a cotransfected plasmid in the same samples. C, The levels of cytosolic β-catenin were measured by Western blotting from LNCaP transfected with 30 ng of the IGF-I receptors and 30 ng of antisense β-catenin plasmids (AS-β-cat). D, One hundred nanograms of *PSA*7kb-luc reporter with or without 30 ng of the β-catenin antisense plasmids were transfected into LNCaP cells. The cells were cultured in T-medium with or without 100 ng/ml of IGF-I in the presence or absence of DHT for 28–32 h. IGF-1R antibody (1 μg/ml) (catalog no. GR11, CalBiochem, San Diego, CA) or mouse normal IgG was added into cells 6 h after transfection. Luciferase and β-gal activities were measured as described above.

IGF-I Stabilizes β-Catenin in Prostate Cancer Cells

It has been shown that IGF-I enhances the stability of the β-catenin protein in human colorectal cancer cells (4). IGF-I inhibits glycogen synthase kinase-3β (GSK3β) by stimulating the phosphorylation of GSK3β (17, 18). We therefore examined whether the IGF-I enhancement of the stability of β-catenin in prostate cancer cells is mediated through GSK3β. Because LiCl has been shown to repress GSK3β (19), thereby stabilizing cellular β-catenin, we measured the cytosolic level of β-catenin in LNCaP cells treated with or without IGF-I. As observed previously, we saw an increase in β-catenin in cells treated with IGF-I (Fig. 4A). In the presence of 50 mM LiCl, the samples isolated from cells treated with 100 ng/ml of IGF-I showed higher levels of β-catenin than ones not treated with LiCl. We conclude that inhibition of GSK3β can further enhance the levels of cellular β-catenin induced by IGF-I in prostate cancer cells.

To demonstrate that the effect of IGF-I was on the stability of β-catenin in prostate cancer cells, we performed pulse-chase experiments in LNCaP cells. Cells were pulsed with Tran^{35}S label and chased in the presence or absence of IGF-I for 12 h. ^{35}S-labeled β-catenin proteins were immunoprecipitated from the cytosolic fractions and analyzed by SDS-PAGE. As shown in Fig. 4B, IGF-I enhances the stability of β-catenin, increasing its half-life from 9–12 h. These results provide a direct line of evidence that IGF-I indeed affects the stability of β-catenin in prostate cancer cells.

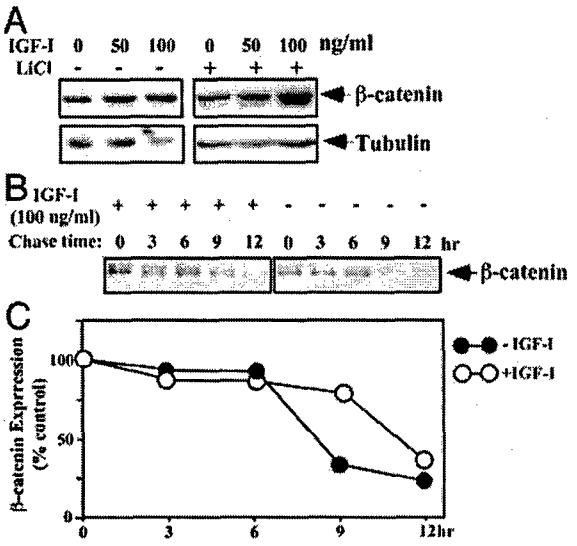

Fig. 4. IGF-I Enhances the Stability of β-Catenin

A, The cytosolic fraction was used to measure the free form of β-catenin in LNCaP cells treated with different concentration of IGF-I in the presence or absence of 50 mM LiCl. B, LNCaP cells were pulsed with Tran^{35}S-label and chased with medium containing an excess of cold methionine/cysteine for the indicated times. The cytosolic fractions isolated from cells were immunoprecipitated for β-catenin. C, The results were analyzed by densitometry and expressed graphically as a percentage of the value at 0 h. The figure shows results of a single experiment, which was repeated once with similar results.

DISCUSSION

Induction of ligand-independent activation of AR by IGF-I and other polypeptide growth factors was initially observed several years ago (3). However, the mechanism(s) by which the growth factors induce AR-mediated transcription were still unknown. It has been shown that IGF-I increases the cellular level of β-catenin in human colon cancer cells (4). In addition, β-catenin has been demonstrated to interact with AR and to enhance AR-mediated transcription (5, 6, 8, 20). The results described here provide multiple lines of evidence demonstrating that β-catenin is involved in the induction by IGF-I of AR-mediated transcription. This was demonstrated by experiments showing that IGF-I enhanced expression of *PSA* in prostate cells, that IGF-I increased the levels of β-catenin at least in part by stabilizing the protein, and finally, that increasing the levels of IGF-1R in prostate cells enhances the level of AR-mediated transcription.

We showed that IGF-I enhances the expression of endogenous *PSA* transcripts in cells treated with low levels of the DHT by both real-time PCR and Northern blot experiments. These results are different from those of Culig *et al.* (3), who showed previously that induction of AR activity by IGF-I on reporters driven by the androgen response element (ARE)- or murine mammary tumor virus promoters was an androgen-independent effect. Obviously, there are several differences between our and their experiments. Although we do not know the exact reason(s) for these differences, we feel that our experiments, which examine the *PSA* transcripts, a natural, endogenous AR-target gene, are more sensitive and biologically relevant for assessing AR activity. It has been shown that the unbound AR forms a complex with heat-shock proteins (21), and upon binding to ligand, the AR dissociates from the heat-shock proteins and translocates into the nucleus (22). Our data agree with the previous observations, suggesting that the IGF-I-induced AR activity requires the nuclear translocation of AR upon binding to ligand, which is also supported by the recent observation that β-catenin augmenting the AR-mediated activity is a nuclear effect and requires the nuclear translocation of AR in the presence of the ligand (6, 8).

LNCaP is currently the only well-characterized prostate cancer cell line that contains both functional AR and E-cadherin pathways and is responsive to IGF-I (23–25). However, it has been shown that there are a relatively low number of IGF-I receptors on these cells, approximately 1×10^4 receptors/cell (25). To examine the effect of having a more physiologically relevant number of receptors on the cells, we transfected wild-type IGF-1R and a constitutively activated mutant of IGF-1R into LNCaP cells. Both the wild-type and the mutant IGF-1R enhanced AR activity from the 7-kb *PSA* promoter in a dose-dependent manner, indicating that IGF signaling modulates AR-mediated transcription. We also performed transient transfection experiments with a luciferase reporter driven by a minimal promoter with two AREs in LNCaP cells. We only observed a moderate effect of IGF-1R on the ARE-reporter, in contrast to the *PSA*-reporter (data not shown). This result may suggest that other transcription factors that bind to sites adjacent to the ARE in the natural *PSA*-promoter may enhance the AR in response to IGF-I-mediated induction.

The cellular levels of β-catenin are tightly regulated in normal cells. Mutations affecting the degradation of β-catenin can result in the accumulation of the cellular β-catenin to induce neoplastic transformation (26). Due to an abnormal cadherin-catenin interaction in the cell membrane, increasing the cytoplasmic and nuclear levels of β-catenin as a consequence of loss of E-cadherin is also frequently observed in late stages of prostate cancer cells (27). In this current study, intriguingly, we demonstrated that IGF-I increases the cellular levels of β-catenin in the prostate cancer cell line, LNCaP, which suggests a novel mechanism by which IGF-I modulates AR mediated transcription. Using both Western blot and pulse-chase assays, we further showed that an increase in β-catenin levels by IGF-I in LNCaP cells is the result of stabilization of β-catenin. Previous studies have shown that GSK3β is one of the major components in the destruction complex that constitutively down-regulates the level of cellular

β-catenin. The signaling mediated by phosphoinositol 3-kinase (PI3K)/Akt can regulate GSK3β through the phosphorylation of the protein (28). The tumor suppressor, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), negatively regulates the PI3K pathway by blocking activation of Akt (29). Our observation that lithium chloride, an inhibitor of GSK3β, enhances the stabilizing effect of IGF-I on β-catenin suggesting a potential link between the IGF-I and PI3K/Akt signaling pathways in modulating Wnt/β-catenin signaling. Our results are also consistent with an earlier report that showed that IGF-I can stabilize the β-catenin protein in combination with lithium chloride in a human colon cancer cell line (4). Further studies of the interaction between IGF, PI3K/Akt, and Wnt/β-catenin pathways may help us to understand their roles in cell-cell adhesion, cell migration, transformation, and tumor metastasis.

In this study, we showed that IGF-I enhances AR-mediated transcription and increases the levels of cellular β-catenin. In addition, we also observed a slight increase in β-catenin in cells treated with 100 ng/ml IGF-II (see Fig. 2B). This result suggests a possible role for IGF-II in the regulation of cellular β-catenin, which is consistent with the recent report that showed that IGF-II induces rapid β-catenin relocation to the nucleus during epithelium to mesenchyme transition (30). To further evaluate the roles of IGF-II and EGF in AR-mediated transcription, we also performed transient transfection experiments using the *PSA*-promoter/reporter in LNCaP cells. We observed a notable induction only by IGF-I in our experiments (data not shown), which is similar to the previous report by Culig *et al.* (3).

In conclusion, in this study we provide several lines of evidence linking IGF-I to the regulation of β-catenin. Because β-catenin has been identified as an AR coregulator, demonstration of a link between IGF-I and β-catenin suggests a potential, novel mechanism by which IGF-I regulates prostate cancer cells in their progression to the androgen-insensitive stage. Further study of this linkage may help us to understand the roles of IGF-I signaling in prostate cancer pathogenesis.

MATERIALS AND METHODS

Real-Time PCR and Northern Blot Analysis

Total RNAs were isolated from LNCaP cells treated with IGF-I in the presence or absence of 0.1 nM DHT by using an RNAwiz kit (Ambion, Austin, TX), and RNA concentration was estimated from absorbance at 260 nm. Expression levels of *PSA* mRNA were quantified using quantitative fluorescent real-time PCR. RNA was first reverse-transcribed using random hexamers as described by the manufacturer (PE Applied Biosystems, Foster City, CA). Two specific primers selected from the regions around the translation initiation site or the stop codon of the *PSA* gene were used for amplification. PCR assays were performed with TaqMan PCR reagent Kits in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The levels of *PSA* mRNA were normalized by coamplification of GAPDH mRNA as described by the manufacturer (PE Applied Biosystems). Northern blotting assays were performed as described previously (31).

Cell Cultures and Transfections

The monkey kidney cell line, CV-1, was maintained in DMEM supplemented with 5% fetal calf serum (FCS) (HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T-medium (Invitrogen Life Technologies, Carlsbad, CA) with 10% FCS. Transient transfections were carried out using LipofectAMINE 2000 (Invitrogen) as described previously (31). In the experiments with IGF-I and other growth factors (Sigma, St. Louis, MO), cells were usually cultured in T-medium for 16 h, and then were treated with different concentrations of growth factors for 20–24 h. For androgen induction experiments, cells were grown in T-medium with charcoal-stripped fetal calf serum (HyClone) for 16–24 h in the presence or absence of DHT.

Whole Cell and Nuclear Extracts

Both whole cell lysate and nuclear extracts were prepared as described previously (6, 32). The cytosolic or cytoskeletal fractions were prepared in digitonin lysis buffer [1% digitonin, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$] or in RIPA buffer [0.5% Nonidet P-40, 0.3% Triton X-100, 15 mM $MgCl_2$, 5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.8)], respectively (4). Protein fractions for immunoblotting were boiled in sodium dodecyl sulfate-sample buffer and then resolved on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with anti-β-catenin antibody (catalog no. C19220, Transduction Labs, Lexington, KY). Proteins were detected using the ECL kit (Amersham, Arlington Heights, IL). The antibody against H1 (Santa Cruz Biotechnology, Santa Cruz, CA) or tubulin (Neomarker, Fremont, CA) was used for protein loading.

Plasmids

The reporter plasmid, p*PSA*7kb-luc, was provided by Dr. Jan Trapman (33). IGF-1R expression vectors were the kind gift of Dr. Weiqun Li (16). The antisense construct of human β-catenin and the pcDNA3-FLAG-β-catenin vector were generated as described previously (6).

Luciferase and β-Galactosidase (β-gal) Assay

Luciferase and β-gal activities were measured as previously described (6, 32). The relative light units from individual transfections were normalized by measurement of β-gal activity expressed from a cotransfected plasmid in the same samples. Individual transfection experiments were done in triplicate and the results are reported from representative experiments.

Pulse-Chase

LNCaP cells were transfected with wild-type pcDNA3-FLAG-β-catenin. After 24 h of transfection, the cells were incubated with DMEM without L-methionine and L-cysteine (Invitrogen Life Technologies) for 1 h, and then pulse-labeled with 100 μCi of Tran ^{35}S Label (ICN, Irvine, CA) for 30 min. The cells were washed twice with PBS and then chased by incubating in complete DMEM in the presence or absence of IGF-I (50 ng/ml) for various periods of time. The cells were lysed in RIPA and digitonin lysis buffers containing protease inhibitors. ^{35}S-labeled β-catenin protein was immunoprecipitated from the cytosolic fractions using an anti-β-catenin rabbit polyclonal antibody (H-102; Santa Cruz Biotechnology) and analyzed by SDS-PAGE.

Statistical Analysis

The difference in the values between two groups was analyzed using the Student's t test. $P < 0.05$ was considered statistically significant.

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Wnt3a Growth Factor Induces Androgen Receptor-Mediated Transcription and Enhances Cell Growth in Human Prostate Cancer Cells

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ABSTRACT

The Wnt signaling pathway plays a critical role in embryogenesis and tumorigenesis. However, biological roles of Wnt growth factors have not been fully characterized in prostate development and the pathogenesis of prostate cancer. In this study, we used Wnt3a-conditioned medium (Wnt3a-CM) and purified Wnt3a proteins to investigate whether there is a direct effect of Wnt3a on androgen receptor (AR)-mediated transcription and to determine its role in the growth of prostate cancer cells. We demonstrated that Wnt3a-CM either induces AR activity in the absence of androgens or enhances AR activity in the presence of low concentrations of androgens, whereas purified Wnt3a showed a pronounced effect in the presence of low concentrations of ligands. We also showed that Wnt3a-CM and the purified Wnt3a enhance the level of cytosolic and nuclear β-catenin, suggesting an involvement of β-catenin in this regulation. Moreover, treatment of LNCaP cells with Wnt3a-CM and purified Wnt3a significantly enhances cell growth in the absence of androgens. Our findings demonstrate that Wnt3a plays an important role in androgen-mediated transcription and cell growth. These results suggest a novel mechanism for the progression of prostate cancer.

INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of cancer deaths in the United States (1). The androgen signaling pathway, which is mainly mediated through the androgen receptor (AR), is important for the normal and neoplastic development of prostate cells (2, 3). Androgen ablation is an effective treatment for the majority of patients with advanced prostate cancer (4). However, most of the patients develop androgen-insensitive prostate cancer within 2 years, for which there is currently no effective treatment. Multiple mechanisms by which prostate cancer cells progress to androgen-insensitive stages have been proposed (3, 5). Recently, several lines of evidence have led to an increased interest in defining the possible role of Wnt signaling in the development and progression of prostate cancer [please see the review by Chesire and Isaacs (6)].

The Wnt ligands, of which there are more than 19 closely related but distinct secreted cysteine-rich glycoproteins, have been characterized according to their roles in early development and tumorigenesis.[4] Evidence from recent studies suggests critical roles for the Wnt ligands in controlling cell proliferation, adhesion, survival, movement, and polarity (7, 8). Receptors for the Wnt proteins are members of the Frizzled family. In vertebrates, Wnt proteins activate different intracellular signaling cascades either through the "canonical" or "non-canonical" pathways (9). In the canonical pathway, secreted Wnt ligands bind to Frizzled and regulate the stability of β-catenin, a key component of Wnt signaling. In the absence of a Wnt signal, β-catenin is constitutively down-regulated by the multicomponent destruction complex containing glycogen synthase kinase 3β, axin, and APC, which promotes phosphorylation on the serine and threonine residues in the NH_2-terminal region of β-catenin and thereby targets it for degradation through the ubiquitin proteasome pathway (10). Wnt signaling inhibits this process, which leads to accumulation of β-catenin in the nucleus and promotes the formation of transcriptionally active complexes with lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF) transcription factors (11, 12).

Wnt signaling and its key component, β-catenin, have been implicated in human malignancy (13, 14). The link between stabilized β-catenin and tumorigenesis was considerably strengthened by discoveries of mutations in the destruction complex and in β-catenin itself in a variety of human tumors (15). Loss of control of intracellular β-catenin levels through mutation in β-catenin itself and/or other components of the protein degradation complex has been reported in prostate cancer samples (16, 17). However, only a small proportion of prostate cancer samples possessed these mutations, suggesting that other possible mechanisms may be involved in the regulation. It has been shown that loss of E-cadherin can result in an increase of the cellular β-catenin in prostate cancer cells (18). Overexpression of E-cadherin in E-cadherin–negative tumor cells decreases cellular β-catenin levels and reduces AR-mediated transcription (19).

A protein–protein interaction between the AR and β-catenin has been identified (19–21). Through this interaction, β-catenin acts as an AR coactivator, augmenting AR-mediated transcription (19). These data provided an additional line of evidence linking Wnt/β-catenin to the androgen signaling pathway in the growth and progression of prostate cancer.

Potential roles for Wnt in tumorigenesis were suggested previously (22, 23). However, the molecular mechanisms by which Wnt signaling regulates the growth and progression of tumor cells are unclear. Knowledge regarding Wnt signaling in the pathogenesis of prostate cancer is lacking. In this study, we examine the role of Wnt 3A in the regulation of androgen signaling in prostate cancer cells. Intriguingly, we demonstrated that Wnt3a induces AR-mediated transcription and cell growth in a ligand-independent manner. These findings provide the first line of evidence that the Wnt growth factor can regulate and interact with the androgen signaling pathway in prostate cancer cells, which suggests a novel mechanism for the development of androgen-insensitive prostate cancer.

MATERIALS AND METHODS

Cell Culture and Conditioned Medium Production. The monkey kidney cell line CV-1 and human prostate cell lines DU145 and PC3 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS; HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T medium (Invitrogen, Carlsbad, CA) with 5% FCS. Wnt3a-conditioned medium (Wnt3a-CM) and L cell control medium (L-CM) were prepared as described previously (24). Briefly, mouse L cells stably transfected with a Wnt3a cDNA driven by the rat phosphoglycerokinase gene promoter were cultured in DMEM supplemented with 10% charcoal-stripped FCS (CS-FCS) for 4 days. The Wnt3a-CM was then harvested, centrifuged at 1,000 × *g* for 15 minutes, and filtered using 0.45 μm cellulose

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[4] http://www.stanford.edu/~rnusse/wntwindow.html.

acetate bottle top filters (Corning, New York, NY). L-CM was prepared under the same conditions from L cells stably transfected with the pGKneo vector alone (24).

Plasmid Construction. The pGL3-OT and pGL3-OF constructs were the gifts of Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). The pcDNA-TCF-1 construct was provided by Dr. H. C. Clevers (Center for Biomedical Genetics, Utrecht, the Netherlands). The pPSA7kb-luc plasmid was obtained from Dr. Jan Trapman (Department of Pathology, Erasmus University, Rotterdam, The Netherlands) (25). A cytomegalovirus-driven β-galactosidase (β-gal) reporter was generated by cloning the lacZ gene into the pcDNA3 vector (19). A double-stranded oligonucleotide corresponding to the human AR cDNA sequence (5′-GGT-GTCACTATGGAGCTCTCA-3′, amino acids 568–575) was synthesized and cloned into the pBS/U6 vector, provided by Dr. Yang Shi (Harvard Medical School, Boston, MA) to make the short hairpin RNA (shRNA) construct (26).

Transfection, Luciferase, and β-gal Assays. Transient transfections were carried out using LipofectAMINE 2000 (Invitrogen). Cells were incubated with Wnt3a-CM or L-CM in the presence or absence of dihydrotestosterone (DHT) 24 hours after transfection. After an 18- to 24-hour incubation, cells were harvested, and the luciferase and β-gal activities were measured. The relative light units (RLU) from individual transfections were normalized using β-gal activity in the same samples. Individual transfection experiments were done in triplicate, and the results are reported as mean RLU/β-gal (±SD).

Preparation of Cell Fractions. LNCaP cells treated with Wnt3a-CM or the control L-CM were grown to confluence in 6-well plates, washed once with PBS, and harvested by scraping. Cells were then centrifuged at 750 × *g* for 2 minutes, resuspended in a hypotonic buffer [10 mmol/L Tris-HCl (pH 7.8), 10 mmol/L KCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin] and incubated on ice for 10 minutes. The cells were lysed by Dounce homogenization and then centrifuged at 2,000 × *g* for 30 minutes to pellet unlysed cells and nuclei. The cytosolic fraction was obtained by further fractionation at 100,000 × *g* for 1 hour.

Northern Blotting. Total RNA from LNCaP cells treated with Wnt3a-CM or L-CM in the presence or absence of DHT was isolated using a RNAwiz kit (Ambion, Austin, TX). Six micrograms of RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham Biosciences, Piscataway, NJ), and hybridized with a DNA fragment (amino acids 1–261) derived from the human prostate-specific antigen (PSA) gene. Hybridization was performed overnight at 65°C in 0.5 mol/L sodium phosphate (pH 7.2), 1% bovine serum albumin, and 7% SDS. The blots were stripped and rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase probe (27).

Immunoprecipitation and Western Blotting. Coimmunoprecipitation assays were carried out essentially as described previously (28). Proteins were eluted by boiling in SDS-sample buffer, resolved by 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. The membranes were then probed with a 1:500 dilution of a polyclonal antibody against the NH_2 terminus of AR (Upstate, Charlottesville, VA) or an anti–β-catenin monoclonal antibody. Proteins were detected using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). The anti-Wnt3a polyclonal antibody was generated and used in the study.

Immunofluorescence. Cells were cultured in 8-well Lab Tek chambered cover slides (Nalge Nunc International, Naperville, IL), fixed in 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were then incubated with anti–β-catenin monoclonal antibody (Signaling Transduction Laboratories, Lexington, KY) for 1 hours and labeled with anti-mouse 594 Alexa secondary antibody (Molecular Probes, Eugene, OR). The nuclei were counterstained with 10 μg/mL Hoechst (Molecular Probes). Samples were analyzed with a Zeis LSM confocal laser scanning microscope.

Cell Proliferation and Colony Formation. Approximately 2,000 cells per well were plated and cultured in the presence of either Wnt3a-CM or L-CM and then harvested at different time points. Proliferation assays were carried out using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) kit (Promega, Madison, WI). Cell numbers were determined by absorbance at 490 nm as suggested by the manufacturer. For colony formation assay, LNCaP cells were plated in 6-well plates (500–1,000 cells per well) for 24 hours and then maintained in Wnt3a-CM or L-CM or in DMEM containing purified Wnt3a proteins for 10 to 12 days. The cells were stained with crystal violet (Sigma, St. Louis, MO), and colonies containing more than 50 cells were counted. Colony assays were performed a minimum of three times, and the results are reported as a mean of three experiments.

Purification of Wnt3a. Purified Wnt3a proteins were isolated as described previously (29). A detailed protocol can also be found on-line.[4]

RESULTS

Wnt3a-CM Enhances AR-Mediated Transcription in a Ligand-Independent Manner. The Wnt pathway has been implicated in the growth and differentiation of various tissues and organs. Recent data showing that β-catenin, a key player in the Wnt pathway, interacts with the AR provided a direct link between Wnt signaling and the pathogenesis of prostate cancer (19–21). In this study, we first used Wnt3a-CM to directly investigate the role of Wnt signaling in prostate cancer cells. Using a specific antibody, we verified the expression of Wnt3a in the Wnt3a-CM prepared from the mouse L cells (Fig. 1*A*). We found that treatment with Wnt3a-CM of both mouse L and DU145 cells increased the level of cytosolic β-catenin (Fig. 1*B*). Moreover, the Wnt3a-CM induced β-catenin–mediated TCF-1 transcription (Fig. 1*C*). These results are consistent with a previous report and confirmed the properties of the Wnt3a-CM (24).

To evaluate the effect of Wnt3a on AR-mediated transcription, we transfected a luciferase reporter driven by the 7-kb PSA gene promoter, an AR-regulated target gene (30), into LNCaP cells. The cells were cultured in the presence or absence of DHT with or without Wnt3a-CM. As shown in Fig. 2*A*, Wnt3a-CM significantly increased endogenous AR-mediated transcription from the PSA promoter. Intriguingly, cells treated with the Wnt3a-CM showed an approximately

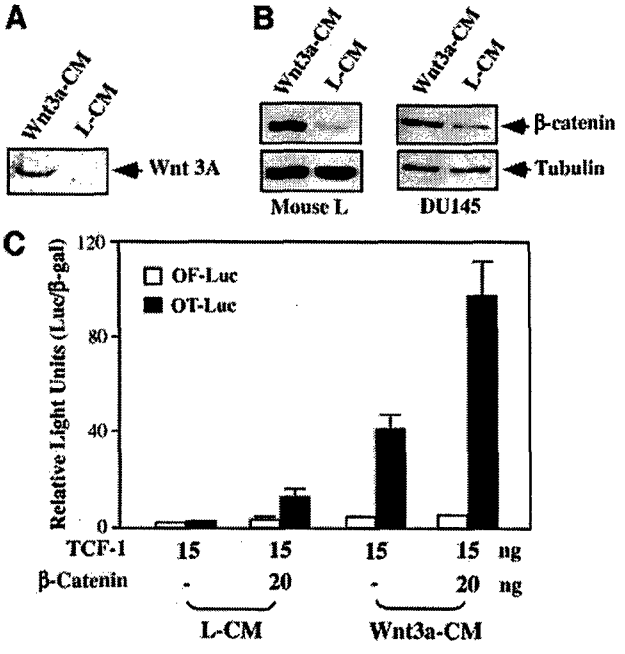

Fig. 1. Wnt3a-CM stabilizes β-catenin and stimulates TCF-mediated transcription. *A*. Ten microliters of Wnt3a-CM and control medium were analyzed by Western blot with a rabbit polyclonal antibody against Wnt3a. *B*. Mouse L and DU145 (a human prostate cancer cell line) cells were cultured with either Wnt3a-CM or L-CM for 20 hours. Whole cell lysates were prepared and analyzed by Western blot with an anti–β-catenin antibody. The samples were also probed with an anti-tubulin antibody to verify equal loading. *C*. CV-1 cells were transiently transfected with 100 ng of pGL3-OT (*OT-Luc*) or the inactive mutant pGL3-OF (*OF-Luc*), 25 ng of pcDNA3-β-gal (*β-gal*), and other plasmids as identified in the figure. The transfected cells were incubated in DMEM with 5% FCS for 24 hours, washed, and then cultured with either Wnt3a-CM or L-CM for another 24 hours. The cells were harvested, and the luciferase and β-gal activities were measured. Luciferase activity is reported as RLU (luciferase/β-gal) and represented as mean ± SD.

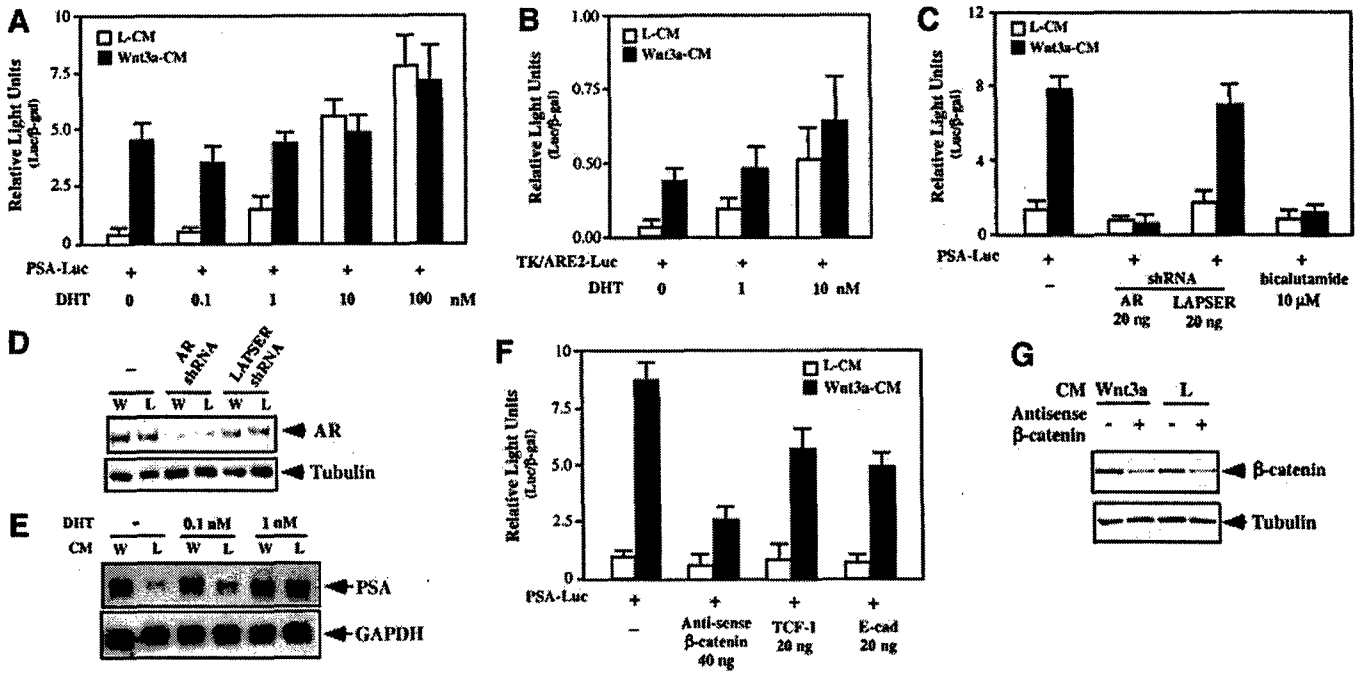

Fig. 2. Wnt3a-CM induces AR-mediated transcription. *A*. LNCaP cells were transiently transfected with 100 ng of PSA7kb-luc reporter (*PSA-Luc*) and 25 ng of pcDNA3-β-gal and incubated in T-medium with 5% CS-FCS for 16 hours. The cells were washed and incubated with Wnt3a-CM or L-CM in the presence of different amounts of DHT for another 24 hours. Luciferase and β-Gal activities were measured and reported as RLU. *B*. LNCaP cells were transfected with 100 ng of TK/ARE2-luc and 25 ng of pcDNA3-β-Gal and treated as described above. Luciferase and β-gal activities were measured. *C*. Transient transfections were carried out as described above. Twenty ng of AR shRNA plasmid or LAPSER shRNA vector (45), as the control, and other plasmids as marked in the figure were transfected into LNCaP cells. Cells were cultured with either Wnt3a-CM or L-CM. An AR antagonist, bicalutamide, was added into cells 24 hours after transfection. Luciferase and β-gal activities were measured. *D*. The cytosolic fractions were prepared from the above-mentioned LNCaP cells transfected with AR or LAPSER shRNA constructs and analyzed by Western blot with AR and tubulin antibodies. *E*. LNCaP cells were cultured with Wnt3a-CM or L-CM, in the absence of DHT or in the presence of 0.1 or 1 nmol/L DHT, for 24 hours. Total RNA was isolated. Six micrograms of RNA were analyzed by Northern blot with a DNA fragment (amino acids 1–261) derived from the human PSA gene. A human glyceraldehyde-3-phosphate dehydrogenase probe (*GAPDH*; amino acids 104–168) was used to verify equal loading. *F*. LNCaP cells were transiently transfected with 100 ng of PSA-Luc, 25 ng of pcDNA3-β-gal, and 40 ng of antisense β-catenin vector or 20 ng of TCF-1 or E-cadherin expression vector. Transfected cells were incubated in T-medium with 5% CS-FCS for 24 hours and then treated as described in *A*. *G*. Cytosolic fractions were isolated from the above-mentioned cells and analyzed by Western blot with β-catenin and tubulin antibodies.

11-fold induction of AR activity compared with cells treated with the control medium in the absence of DHT. These results provide the first evidence that the Wnt growth factor can independently activate AR-mediated transcription. In addition, Wnt3a-CM also showed an induction of AR-mediated transcription in the presence of 0.1 and 1 nmol/L DHT.

To verify that induction of the PSA promoter by Wnt3a-CM is a specific effect, we repeated the experiments in LNCaP cells with a luciferase reporter driven by a minimal promoter with two androgen response elements (AREs). A similar induction of AR-mediated transcription was observed in the cells treated with Wnt3a-CM (Fig. 2*B*). To further ensure that the induction by Wnt3a-CM is directly through the AR protein, we tested the effect of Wnt3a-CM on cells that were cotransfected with a shRNA construct of AR to knock down the AR protein. As shown in Fig. 2*C*, reduction of AR protein expression can abolish the AR-mediated transcription. This was correlated with a decreased level of cytosolic AR proteins in the cells (Fig. 2*D*). In addition, an AR antagonist, bicalutamide, can also block the activity of AR in cells treated with Wnt3a-CM (Fig. 2*C*). Taken together, these data indicate that the effect of Wnt3A is mediated through AR.

To evaluate the effect of Wnt3a-CM in a biologically relevant setting, we tested whether the conditioned medium regulates expression of the endogenous PSA gene. We measured transcripts of PSA in LNCaP cells treated with different amounts of DHT in the presence of Wnt3a-CM or control medium. As shown in Fig. 2*E*, Wnt3a-CM induces an approximately 5-fold increase in the expression of PSA in the absence of DHT. In addition, in the presence of 0.1 nmol/L DHT, the expression of PSA is about 2.5-fold higher in cells treated with Wnt3a-CM than in cells treated with L-CM (Fig. 2*E*). These results provide an additional line of evidence that Wnt3a-CM can activate AR-mediated transcription. Taken together, we have demonstrated that Wnt3a-CM can activate AR-mediated transcription in the absence of ligand and augment AR activity in the presence of a low concentration of androgens.

Given that β-catenin is a key downstream effector of the Wnt pathway and acts as an AR coactivator, we further investigated whether β-catenin is involved in the Wnt3A-induced AR activity. We repeated the transient transfection experiments with an antisense construct of β-catenin. As observed previously (19), it specially reduces the level of cellular β-catenin proteins (Fig. 2*G*), and the induction of AR activity by Wnt3a-CM (Fig. 2*F*). It has been shown that overexpression of TCF/LEF and E-cadherin can compete for β-catenin binding to AR and reduce AR-mediated transactivation (31, 32). We therefore tested whether coexpression of TCF-1 and E-cadherin can affect the induction of AR activity mediated by Wnt3a-CM. As shown in Fig. 2*F*, PSA promoter/reporter activities were reduced approximately 35% to 45%, relative to the controls, in cells transfected with the TCF-1 and E-cadherin expression vectors. These data suggest an involvement of β-catenin in the induction of AR activity mediated by Wnt3a-CM.

Wnt3a-CM Increases the Level of Cytosolic and Nuclear β-Catenin in Prostate Cancer Cells. Wnt3a-CM has been shown to increase accumulation of cytosolic free β-catenin (24). Cotransfections of β-catenin antisense and TCF-1 and E-cadherin constructs suggested that β-catenin is involved in Wnt3a-CM–mediated AR activity. To evaluate whether Wnt3a-CM affects the cytosolic pool of β-catenin in LNCaP cells, we examined the levels of β-catenin in the different cellular fractions prepared from cells treated with the

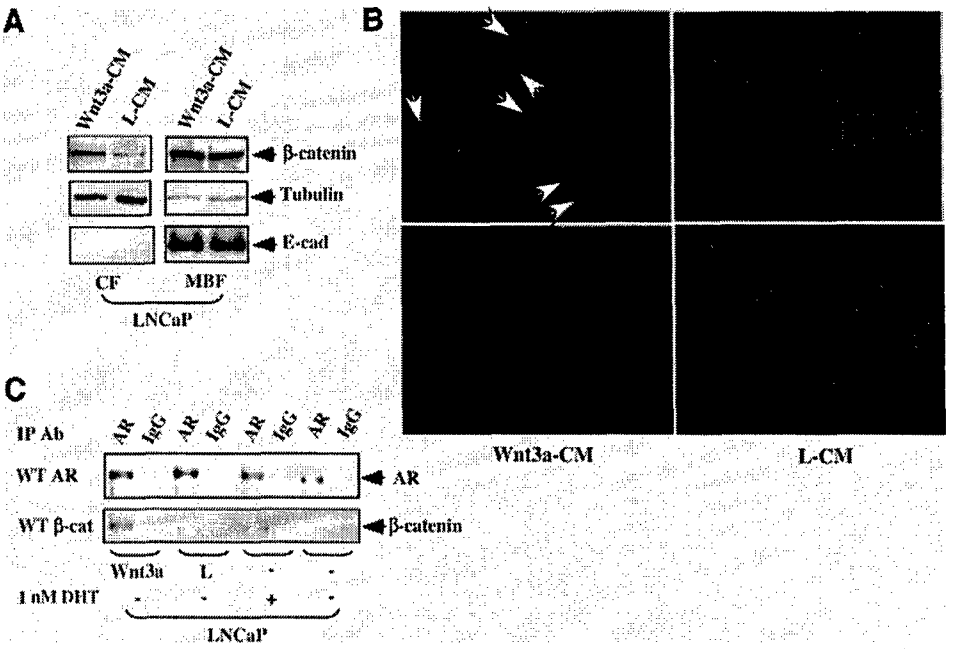

Fig. 3. Wnt3a-CM enhances the level of cytosolic and nuclear β-catenin. *A*. LNCaP cells were cultured in Wnt3a-CM or L-CM for 24 hours and then harvested. A cytosolic fraction (*CF*) and membrane-associated fraction (*MBF*) were prepared and analyzed by Western blotting (see Materials and Methods). The expression of E-cadherin was examined to evaluate the purity of the above-mentioned fractions. *B*. LNCaP cells were cultured in Wnt3a-CM or L-CM for 24 hours, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were stained with the anti–β-catenin antibody followed by a secondary antibody conjugated with rhodamine (*red*). The nuclei were counterstained with Hoechst, and the ones with a high level of β-catenin are marked with *arrows*. *C*. Equal amounts of nuclear fractions isolated from LNCaP cells incubated with Wnt3a-CM (*Wnt3a*) or L-CM (*L*) were subjected to immunoprecipitation with normal mouse IgG or anti-AR monoclonal antibody. The nuclear fractions isolated from LNCaP cells cultured in the presence or absence of 1 nmol/L DHT were also included in experiments as controls. The precipitated fractions were then resolved by SDS-PAGE and analyzed by Western blot using anti–β-catenin or anti-AR antibodies (*WT*).

Wnt3a-CM and control medium. The cytosolic fraction and the membrane-associated fraction were prepared, representing the free cytosolic pool and membrane bound β-catenin, respectively (33). As shown in Fig. 3*A*, there was no significant change in the amount of β-catenin protein in the membrane-associated fraction isolated from the cells treated with Wnt3a-CM in comparison with untreated cells. However, there was a significant increase of cytosolic β-catenin in the cells treated with Wnt3a-CM compared with the controls. In addition, the level of tubulin, as a control, was similar in cells treated with Wnt3a-CM and L-CM in both the cytosolic fraction and membrane-associated fractions. The results indicate that Wnt3a-CM increased the levels of cytosolic β-catenin in LNCaP cells.

Next, we examined the effects of Wnt3a-CM on the cellular localization of β-catenin in LNCaP cells by immunofluorescence staining. As shown in Fig. 3*B*, clear cell membrane staining with the β-catenin antibody was observed in LNCaP cells. However, there is also an increase in nuclear β-catenin in cells treated with Wnt3a-CM. These data are consistent with the results from the Western blot (Fig. 3*A*) and suggest that Wnt3a-CM can stimulate nuclear translocation of β-catenin.

It has been shown that β-catenin forms a protein complex with AR and enhances AR-mediated transcription in LNCaP cells. Given that Wnt3a-CM enhances cytosolic free β-catenin and nuclear translocation of β-catenin, we next examined whether Wnt3a-CM enhances the formation of the β-catenin–AR protein complex in nuclei. Using the nuclear fraction of LNCaP cells treated with Wnt3a-CM, we assessed the levels of β-catenin in the protein complex with AR by coimmunoprecipitation. We observed more β-catenin proteins interacting with AR in cells treated with Wnt3a-CM than in cells treated with the control medium in the absence of 1 nmol/L DHT (Fig. 3*C*). As described previously, we also observed that β-catenin forms a protein complex with the AR in LNCaP cells in the presence of androgens. The data provide another line of evidence indicating that Wnt3a-CM induces the formation of AR–β-catenin protein complexes in the nucleus.

Wnt3a-CM Promotes Cell Growth and Colony Formation in the Absence of Androgens. Next, we investigated the role of Wnt3a-CM in the regulation of LNCaP cell growth. In particular, we addressed whether Wnt3a-CM can function as a growth factor to promote LNCaP cell growth in a ligand-independent manner. LNCaP cells were cultured with Wnt3a-CM that was prepared in RPMI 1640 with 10% CS-FCS (see Materials and Methods). The growth of LNCaP cells was first assessed by the MTS assay. In the presence of Wnt3a-CM, the cell numbers were 20% and 35% higher after 4 and 6 days compared with controls (Fig. 4*A*). We then assessed the growth-promoting effect of Wnt3a-CM using a colony formation assay. Approximately 500 LNCaP cells were seeded in each well and incubated with Wnt3a-CM. After 12 days, cells were fixed and stained with crystal violet. There are more and larger colonies in the samples incubated with Wnt3a-CM than in the ones treated with control medium (Fig. 4*B*). The number of colonies containing >50 cells is significantly higher in the samples treated with Wnt3a-CM than in the controls ($P < 0.001$; Fig. 4*C*). The above-mentioned data demonstrate that Wnt3a-CM increases the growth of prostate cancer cells in the absence of androgens. We also performed the above experiments in the presence of 0.1 nmol/L DHT and observed a clear effect of Wnt3a-CM in enhancing the growth of prostate cancer cells (data not shown).

Purified Wnt3a Proteins Enhance AR-Mediated Transcription and Cell Growth. Recently, Wnt molecules, including the product of the mouse Wnt3a gene, have been isolated (29) and appear active in inducing self-renewal of hematopoietic stem cells. We found that, like Wnt3a-CM, purified Wnt3a proteins can enhance the level of cytosolic β-catenin in two prostate cancer cell lines, PC3 and LNCaP (Fig. 5*A*). Then, we tested the activity of purified Wnt3a proteins in transient transfection assays. The PSA-luc plasmids were transfected with or without a wild-type AR expression vector into PC3 cells, which are AR negative. In the presence of 0.1 nmol/L DHT, Wnt3a-CM induces approximately 35% to 40% of AR-mediated transcription (Fig. 5*B*). Intriguingly, under a similar experimental condition, purified Wnt3a proteins show a potent and dosage-dependent enhancement of AR transactivation. To confirm this finding, we repeated transient transfection experiments in LNCaP cells. As shown in Fig. 5*C*, purified Wnt3a proteins show a similar induction of AR-mediated transcription in the presence or absence of 0.1 nmol/L DHT. However, the effect is more pronounced in the cells treated with the ligand. To further assess the growth-promoting effect of purified Wnt3a proteins, we repeated the colony formation assays. We ob-

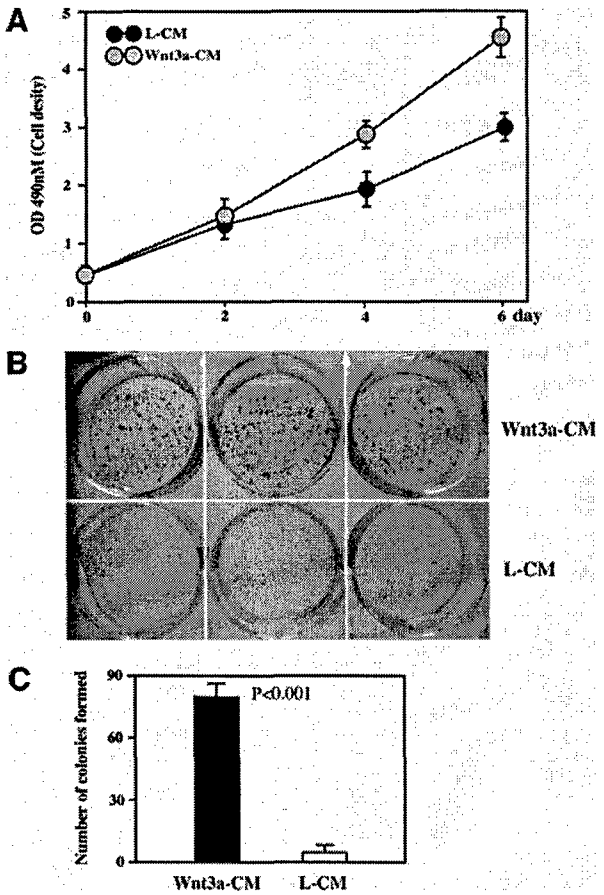

Fig. 4. Wnt3a-CM promotes the growth of LNCaP cells in a ligand-independent manner. *A*. LNCaP cells were cultured with Wnt3a-CM or L-CM in the absence of DHT. At the indicated time points, cells were harvested and analyzed by the MTS assay. The data represent the mean ± SD of three independent experiments. *B*. For the colony formation assay, 500 LNCaP cells were seeded in 6-well plates and cultured in Wnt3a-CM or L-CM in the absence of DHT. Cells were fixed and stained with crystal violet after a 12-day incubation. *C*. Colonies containing >50 cells were counted and analyzed. The results are from three separate transfection experiments.

served an increase of colony size and number in the samples treated with purified Wnt3a proteins compared with the ones treated with buffer only (Fig. 5*D*). The number of colonies containing >50 cells is significantly higher in the samples treated with purified Wnt3a proteins than in the controls ($P < 0.001$; data not shown). Taken together, the above results confirm an important role of the Wnt3a proteins in AR-mediated transcription and prostate cell growth.

DISCUSSION

Wnt signaling pathways regulate a variety of processes including cell growth, development, and oncogenesis (13, 34). However, the biological roles of the Wnt growth factors serving as the upstream signaling of β-catenin have not been fully characterized in prostate cancer cells. In this study, we investigate whether there is a direct effect of the Wnt growth factor on AR-mediated transcription and its role in the growth of prostate cancer cells.

Wnt3a-CM prepared from mouse L cells stably transfected with mouse Wnt3a cDNA has been well characterized (24). It has become a great resource and is used frequently to study the Wnt signaling pathway (8, 35). Previous studies have shown that Wnt3a-CM increases the cytosolic and nuclear levels of β-catenin (8, 24). Microarray data demonstrated that treatment of human embryonic carcinoma with Wnt3a-CM up-regulated the expression of β-catenin, downstream target genes of TCF/LEF, and other factors involved in the regulation of β-catenin (35). These multiple lines of evidence confirm a specific signaling pathway mediated by Wnt3a-CM in cells.

In this study, we showed that Wnt3a-CM stimulates AR-mediated transcription. We demonstrated that Wnt3a-CM is capable of inducing AR-mediated transcription from the PSA promoter/reporter and the expression of endogenous PSA transcripts in a ligand-independent manner. In LNCaP cells, the stimulation by Wnt3a-CM of AR is very effective and is almost as great as the effect achieved by adding 1 nmol/L DHT. In addition, our data also showed that Wnt3a-CM is able to increase AR-mediated transcription in the presence of low concentrations of DHT. These data provide the first line of evidence showing a unique and important role of Wnt3a in the regulation of the androgen signaling pathway in a ligand-dependent manner.

To understand the molecular mechanism by which Wnt3a augments AR-mediated transcription, we performed several experiments to confirm the involvement of the AR in the regulation. We showed that Wnt3a-CM induces transcription of the 7-kb PSA promoter-luc and endogenous PSA gene. Moreover, Wnt3a-CM also affects a minimal promoter containing only two AREs. Furthermore, we demonstrated that induction of AR-dependent promoters by Wnt3a-CM can be completely abolished by an AR shRNA construct and an AR antagonist, bicalutamide. These data implicate that induction by Wnt3a-CM is mediated through the AR.

β-Catenin plays a central role in the Wnt signaling pathway. As reported previously, we observed an increase in free cytosolic and nuclear β-catenin in both prostate and nonprostate cancer cells that were treated with Wnt3a-CM (Fig. 1*A* and *B*). Previous studies by us and others have shown that β-catenin is a coactivator of AR (19–21). Therefore, we examined whether β-catenin is a downstream effector of Wnt3a, augmenting AR-mediated transcription. Using an antisense construct of β-catenin, we were able to partially block the effect of Wnt3a-CM on AR-mediated activity. Moreover, overexpression of TCF or E-cadherin, β-catenin–binding proteins, also reduced the Wnt3a-CM–mediated AR activity on the PSA promoter. These data suggest an involvement of β-catenin in Wnt3a-CM–induced AR transcription, which is in agreement with the previous finding that β-catenin acts as an AR coactivator (19–21). However, given the fact that expression of the antisense β-catenin and TCF and E-cadherin constructs only partially blocks the effect of Wnt3a-CM, it appears that other factors and/or pathways may also be involved in the regulation. It has been shown that Wnt growth factors/ligands can stimulate both canonical and non-canonical pathways. In this regard, the molecular mechanism(s) by which Wnt growth factors regulate the androgen signaling in prostate cells must be explored further.

Although the mechanisms by which prostate cancer cells develop into the androgen-insensitive stage are currently unclear, it is believed that the tumor cells must either bypass or adapt the androgen signaling pathway to survive in a low-androgen environment during progression. AR mutations have been identified in some androgen-insensitive prostate cancers (36–38). Amplification of the *AR* gene has been observed in some biopsy samples during androgen ablation therapy (39). Recent studies showed that the modulation of the AR protein by phosphorylation, acetylation, and sumoylation also regulates AR activity (40–43). In particular, it has been shown that phosphatidylinositol 3′-kinase/Akt and PTEN regulate AR-mediated transcription through either direct phosphorylation of AR proteins (44) or modification of AR cofactors, such as β-catenin (27). In the current study, we provide several lines of evidence demonstrating that Wnt3a is able to stimulate AR-mediated transcription in the absence of ligand or the presence of a low level of ligand in prostate cancer cells. The above-mentioned data suggest that signals delivered through the AR are still essential in androgen-insensitive prostate cancer cells. Intriguingly,

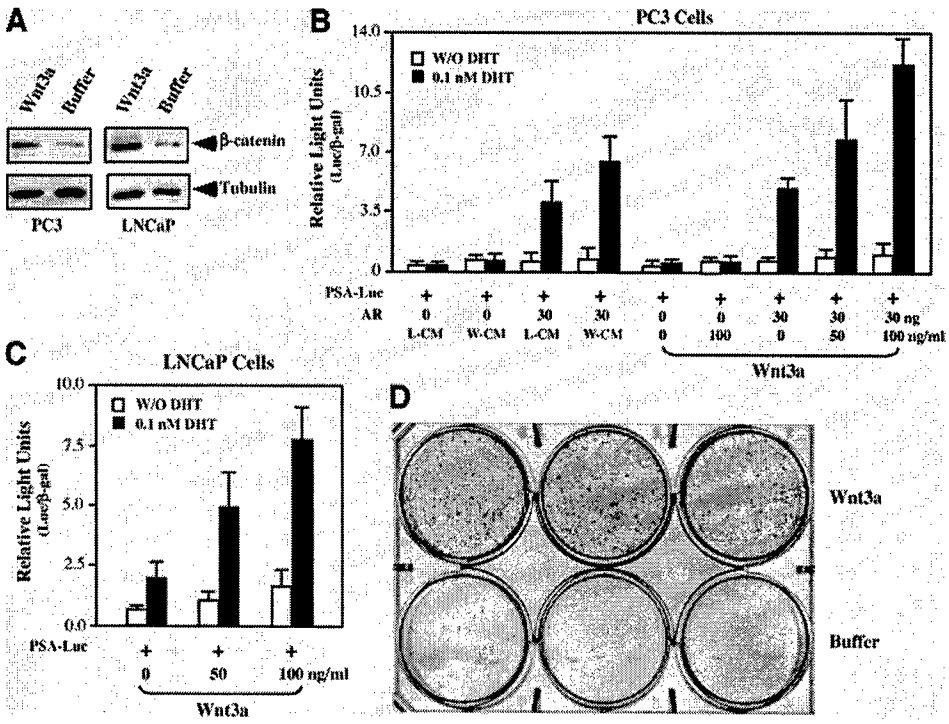

Fig. 5. Purified Wnt3a proteins enhance AR-mediated transcription and cell growth. *A*. Both PC3 and LNCaP cells were cultured in the presence of purified Wnt-3a (100 ng/mL) or the elution buffer (*Buffer*) as a control for 24 hours. Cytosolic fractions were isolated and analyzed by Western blot. *B*. PC3 cells were transiently transfected with 100 ng of PSA-Luc, 25 ng of pcDNA3-β-Gal, and 30 ng of pcDNA3-AR; washed after 24 hours; and then incubated in Wnt-3a-CM or L-CM or treated with the indicated amounts of purified Wnt-3a proteins in the presence or absence of 0.1 nmol/L DHT. Luciferase and β-Gal activities were measured as described previously. *C*. LNCaP cells were transiently transfected with 100 ng of PSA-Luc and 25 ng of pCMV-β-Gal. After 24 hours, transfected cells were washed and incubated with the indicated amounts of purified Wnt-3a. *D*. Approximately 1,000 LNCaP cells were plated in triplicate in DMEM containing 10% CS-FCS in the presence of 100 ng/mL Wnt-3a. Ten days after incubation, the cells were stained with crystal violet. A representative plate is shown. Colonies containing >50 cells were counted and analyzed as described in Fig. 4*C*.

we also show that Wnt3a-CM is able to promote the growth of prostate cancer cells in a ligand-independent manner. The fact that Wnt3a-CM can promote cell growth and induce AR-mediated transcription suggests a unique role of the Wnt growth factor in the progression of prostate cancer cells from the androgen-sensitive to -insensitive stages. It is possible that aberrant expression of Wnt growth factors and/or their receptors in prostate cancer tissues may play a critical role in the progression of prostate cancer.

Although attempts to purify Wnt proteins have been hampered by several technical difficulties, including their high degree of insolubility, active Wnt molecules, including the product of the mouse Wnt3a gene, have been isolated recently (29). In this study, we first showed that, like Wnt3a-CM, purified Wnt3a proteins increase the cytosolic level of β-catenin in prostate cancer cells. Then we confirmed the role of purified Wnt3a proteins on AR-mediated transcription and cell growth in prostate cancer cells. In PC3 cells, Wnt3a proteins enhance AR-mediated transcription in a dosage-dependent manner. A similar effect by purified Wnt3a proteins was also observed in LNCaP cells. These data provide a direct line of evidence demonstrating a true effect of Wnt3a on AR-mediated transcription in prostate cells. However, we observed that purified Wnt3a proteins only slightly affect AR activity in the absence of DHT. This is different from the results that we observed in the experiments when Wnt3a-CM was used. Currently, we do not know the exact reason(s) why purified Wnt3a proteins have less effect on AR-mediated transcription than Wnt3a-CM in the absence of androgens. Further characterization of different protein fractions during purification processes may lead to the identification of additional factors or cofactors that contribute to Wnt3a-mediated augmentation of AR activity. Additional studies of the expression profiles of Wnt ligands and receptors in prostate tissues and prostate cancer cells will also help us to fully understand the signaling pathway(s) regulated by Wnt growth factors in prostate cancer cells.

In this study, we provide several lines of evidence that Wnt3a acts as an upstream signal to induce the transcriptional activity of AR and the growth of prostate cancer cells, possibly through β-catenin. In particular, using purified Wnt3a proteins, we confirm the important role of the Wnt3a growth factor in inducing AR-mediated transcription and cell growth. The effect of Wnt3a may play a critical role in maintaining or increasing AR activity in the setting of decreased androgen levels during androgen ablation therapy. Therefore, further study of the molecular mechanisms by which Wnt growth factors modulate androgen signaling should provide fresh insight into the progression of prostate cancer, which may help us to identify new steps that can be targeted for prostate cancer treatment.

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